

A reproducible mouse model of chronic allograft nephropathy with vasculopathy

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Although short-term outcomes in kidney transplantation have improved dramatically, long-term survival remains a major challenge. A key component of long-term, chronic allograft injury in solid organ transplants is arteriosclerosis characterized by vascular neointimal hyperplasia and inflammation. Establishing a model of this disorder would provide a unique tool not only to identify mechanisms of disease but also to test potential therapeutics for late graft injury. To this end, we utilized a mouse orthotopic renal transplant model in which C57BL/6J (H-2b) recipients were given either a kidney allograft from a completely mismatched Balb/cJ mouse (H-2d) or an isograft from a littermate. A unilateral nephrectomy was performed at the time of transplant followed by a contralateral nephrectomy on post-transplant day 7. Recipients were treated with daily cyclosporine subcutaneously for 14 days and then studied 8 and 12 weeks post transplantation. Renal function was significantly worse in allograft compared with isograft recipients. Moreover, the allografts had significantly more advanced tubulointerstitial fibrosis and profound vascular disease characterized by perivascular leukocytic infiltration and neointimal hyperplasia affecting the intrarenal blood vessels. Thus, we describe a feasible and reproducible murine model of intrarenal transplant arteriosclerosis that is useful to study allograft vasculopathy.

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Despite impressive advances in the initial success rate and short-term survival in clinical transplantation, as well as an impressive body of knowledge of the pathogenesis of graft failure, the rate of long-term graft survival has not significantly improved.^{1–4} Transplant arteriosclerosis is recognized as a significant long-term complication of renal and nonrenal solid organ transplantation.^{5,6} Several studies have indicated both immunologic and nonimmunologic factors that result in endothelial injury and ultimately lead to vascular inflammation, neointima formation, and progressive luminal obstruction.^{7–10} Nevertheless, the exact underlying pathophysiological events remain to be identified. Incomplete understanding of the molecular mechanisms of allograft vasculopathy has hampered our efforts to overcome this detrimental condition. A limited number of animal models exist to study transplant arteriosclerosis, and none has been described using mice in the context of kidney transplantation. Previous studies describing murine models of chronic allograft nephropathy using various strain combinations have not described intrarenal vascular lesions that resemble human transplant arteriosclerosis.

This report describes a mouse orthotopic renal transplant model with striking intrarenal vasculopathy that can be used to study the underlying mechanism and potential therapeutic targets of renal allograft-related vasculopathy.

RENAL FUNCTION IS WORSE IN ALLOGRAFTS

Animal survival and renal function was significantly worse in the allograft recipients compared with the isograft recipients (functional decline was significant in the allograft recipient group starting at 3 weeks post transplantation) (Figure 1a and b). In this model, the outcome of the surgery is highly dependent on the technical skills of the microsurgeon performing the procedure. Of 173 orthotopic kidney transplants performed at our center, the overall survival rate for all kidney transplants was ~78%, with a follow-up of 60 days, much in line with other reported outcomes.^{11,12} These survival data include all animals in which surgery was performed, not just those that survived beyond a given time point, and therefore represent routine, reproducible survival. In all, 21% of the transplant animals died at less than 30 days post transplantation. Reasons for loss of grafts included

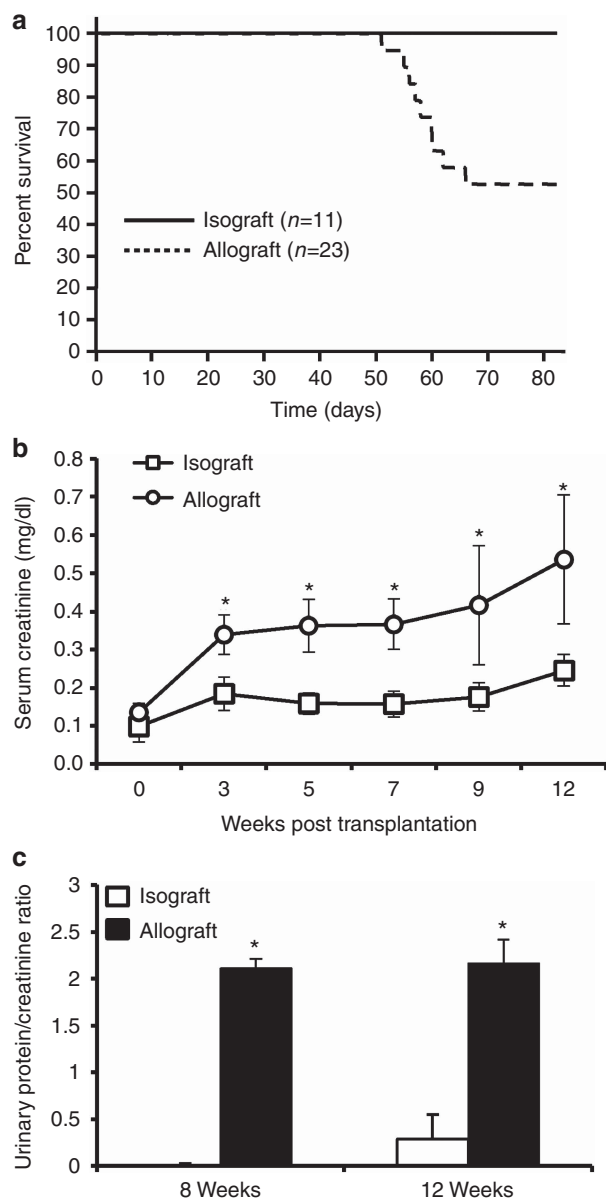


Figure 1 | Survival and renal function in allograft and isograft recipients. (a) Survival rate was monitored after transplantation for 12 weeks. (b) Serum was separated from blood that was collected via retro-orbital puncture, and serum creatinine was determined by LC-MS/MS at the indicated time points ($n = 7/\text{group}$, $*P < 0.05$). (c) Urine was collected at the time of killing, and urinary albumin and creatinine were determined. Data are represented as urinary albumin/creatinine ratio ($n = 7/\text{group}$, $*P < 0.01$).

bleeding from vascular anastomotic site, graft hydronephrosis, and thrombosis. Importantly, there were no differences in terms of technical failures between allografts and isografts. Compared with the isograft recipients, proteinuria (measured by urinary albumin/creatinine ratio) was higher (P -value < 0.05) at 8 and 12 weeks post transplantation in the allograft recipient group (Figure 1c). These results confirmed that mismatched renal allograft recipients develop impaired renal function and proteinuria and reduced survival when compared with isograft recipients.

ALLOGRAFT KIDNEYS DISPLAY INCREASED COLLAGEN DEPOSITION, PERIVASCULAR INFILTRATE, AND NEOINTIMAL HYPERPLASIA

Histological analysis revealed that isografts had better preserved kidney architecture and normal intrarenal arteries (Figure 2a and b, left panels) compared with allografts that displayed increased fibrosis and intrarenal vascular disease (Figure 2a and b, right panels). Semiquantitative analysis revealed markedly higher amounts of interstitial collagen deposition in the allografts at both 8 and 12 weeks post transplantation (Figure 2c). Importantly, intrarenal blood vessels had profound perivascular mononuclear leukocytic infiltration, as well as neointimal hyperplasia characteristic of transplant arteriosclerosis (Figure 3a and b, right panels); these findings were not observed in arteries of the isografts (Figure 3a and b, left panels). Immunophenotyping revealed that the perivascular leukocytic infiltrate is mainly composed of macrophages (CD11b $^{+}$) and cytotoxic CD8 $^{+}$ T cells (Supplementary Figure S1 online). Neointimal hyperplasia was quantified in intrarenal arteries and was higher (P -value < 0.05) in the allografts at 8 and 12 weeks post transplantation compared with isografts (Figure 3c). Applying the Banff schema used for human kidney allografts, the vascular lesions in all allografts at 12 weeks demonstrated neointima formation, significant arterial intimal fibrosis, and mononuclear cell infiltration consistent with Banff grade 3 acute cellular rejection, as well as evidence of chronic active T-cell-mediated rejection (chronic allograft arteriopathy). Furthermore, there was moderate (grade II) interstitial fibrosis and tubular atrophy in the cortical area of the allograft recipients.

In this study, we report a feasible and reproducible model of mouse kidney transplantation with consistent perivascular inflammation and transplant arteriosclerosis. C57BL/6J (H-2 b) recipients received a kidney either from a Balb/cJ mouse (H-2 d ; allograft) or from a littermate (isograft). This reproducibility is based on several quantitative measures including survival data (Figure 1a), assessment of renal function (Figure 1b), proteinuria (Figure 1c), and histological changes (Figures 2a–c and 3a–c). Each of these criteria was quantified as described in the methods and statistical significance. All the allograft recipients (100%) that were evaluated at the 8- and 12-week time point displayed significant vascular neointimal inflammation.

A hallmark lesion of long-term graft loss particularly in solid organs such as the kidney is transplant arteriosclerosis affecting intrarenal blood vessels. Although specific combinations of rat strains are able to reproduce the classic lesion of transplant arteriosclerosis with fibrointimal hyperplasia,¹³ mouse models have not been successful owing to variable outcomes.¹⁴ The model described in this work showing significant intrarenal vascular lesions offers an opportunity for further mechanistic studies to uncover the pathogenesis of this lesion and explore potential therapies. The factors contributing to the development of the vascular lesions seen in this model remain to be determined. It is possible that administration of cyclosporine contributed to the vascular

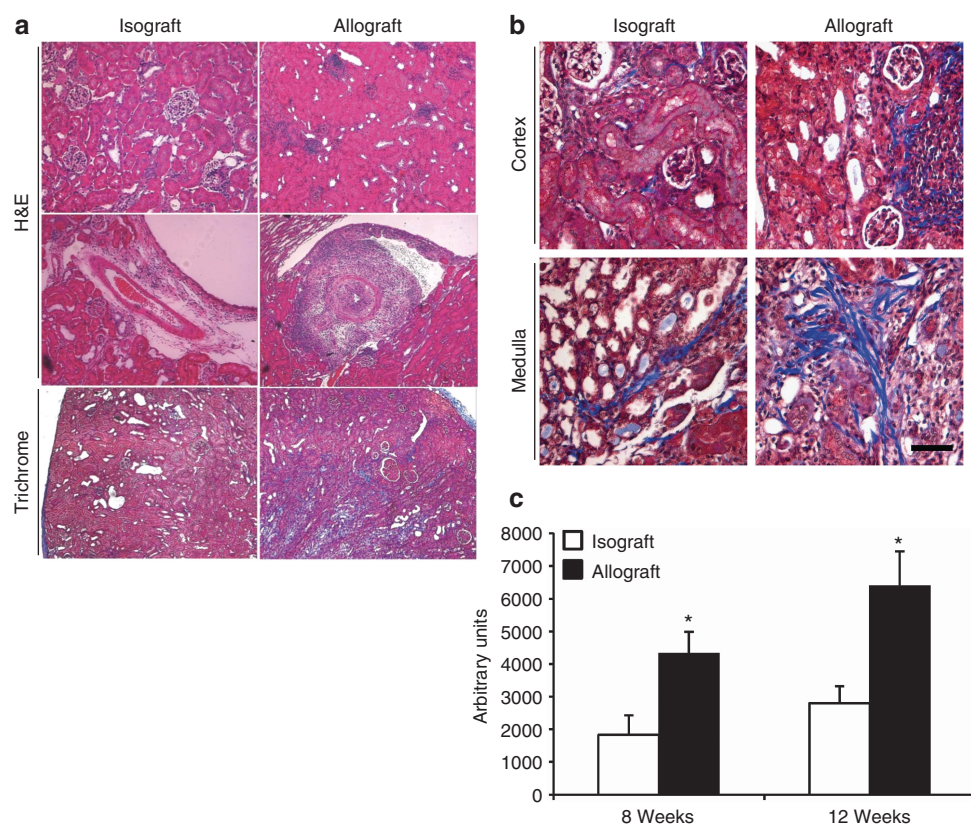


Figure 2 | Increased renal interstitial fibrosis in the allograft recipient group. (a) Histological analysis of renal isografts (left panels) and allografts (right panels). Isografts demonstrate preserved architecture (upper panels), normal intrarenal arteries (middle panels), and less fibrosis (lower panels). (b) Higher magnification of Masson trichrome staining demonstrates the collagen deposition in the cortex (upper panels) and medulla (lower panels). (c) Ten random fields (five cortical and five medullary) were selected from each graft recipient for digital quantification of collagen deposition ($n = 7/\text{group}$, $*P < 0.05$). Bar = 100 μm . H&E, hematoxylin and eosin.

lesions in the allografts, although isografts received similar doses of cyclosporine and did not develop any vascular pathology. It is interesting to note that the blood vessels with marked neointimal hyperplasia also had significant mononuclear infiltration (mainly composed of macrophages (CD11b+) and cytotoxic CD8+ T cells), corroborating previous findings^{15–17} and suggesting that vascular inflammation may be linked to the development of neointimal hyperplasia.

In summary, we report a mouse model of renal transplantation between C57BL/6J (H-2^b) and mismatched Balb/cJ mouse donor (H-2^d; allograft) with high reproducibility and low variability. The findings of vascular lesions of transplant arteriosclerosis in this model would allow the study of pathways involved and hence provide a tool to facilitate translational studies to enhance long-term graft survival.

KIDNEY TRANSPLANTATION

The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Vascularized, orthotopic kidney transplants were performed in mice as previously described.^{18,19} Briefly, donor and recipient male mice weighing between 20 and 25 g were

anesthetized with isoflurane, and the donor kidney, ureter, and bladder were harvested en bloc, including the renal artery with a small aortic cuff and the renal vein with a small caval cuff (Supplementary Video files S1 and S2 online, and Figure 4). The arterial and venous cuffs attached to the donor organ were anastomosed to the recipient abdominal aorta and vena cava, respectively, below the level of the native renal vessels. Donor and recipient bladders were attached dome to dome. The left native kidney was removed at the time of transplant, and the right native kidney was removed through a flank incision 1 week later; care was taken to preserve the recipient adrenal glands with their normal blood supply. Two experimental groups were evaluated. In the isograft group C57BL/6J (H-2^b) recipients received a kidney from a littermate and in the allograft group C57BL/6J (H-2^b) recipients received a kidney from a completely mismatched Balb/cJ mouse (H-2^d; allograft). Both allograft and isograft recipients received cyclosporine at a dose of 10 mg/kg daily subcutaneously for 14 days to prevent acute rejection.

MEASUREMENT OF KIDNEY TRANSPLANT FUNCTION

Renal function was determined by collecting retro-orbital blood at the indicated time points. Serum creatinine levels were measured as previously described using LC-MS/MS.²⁰

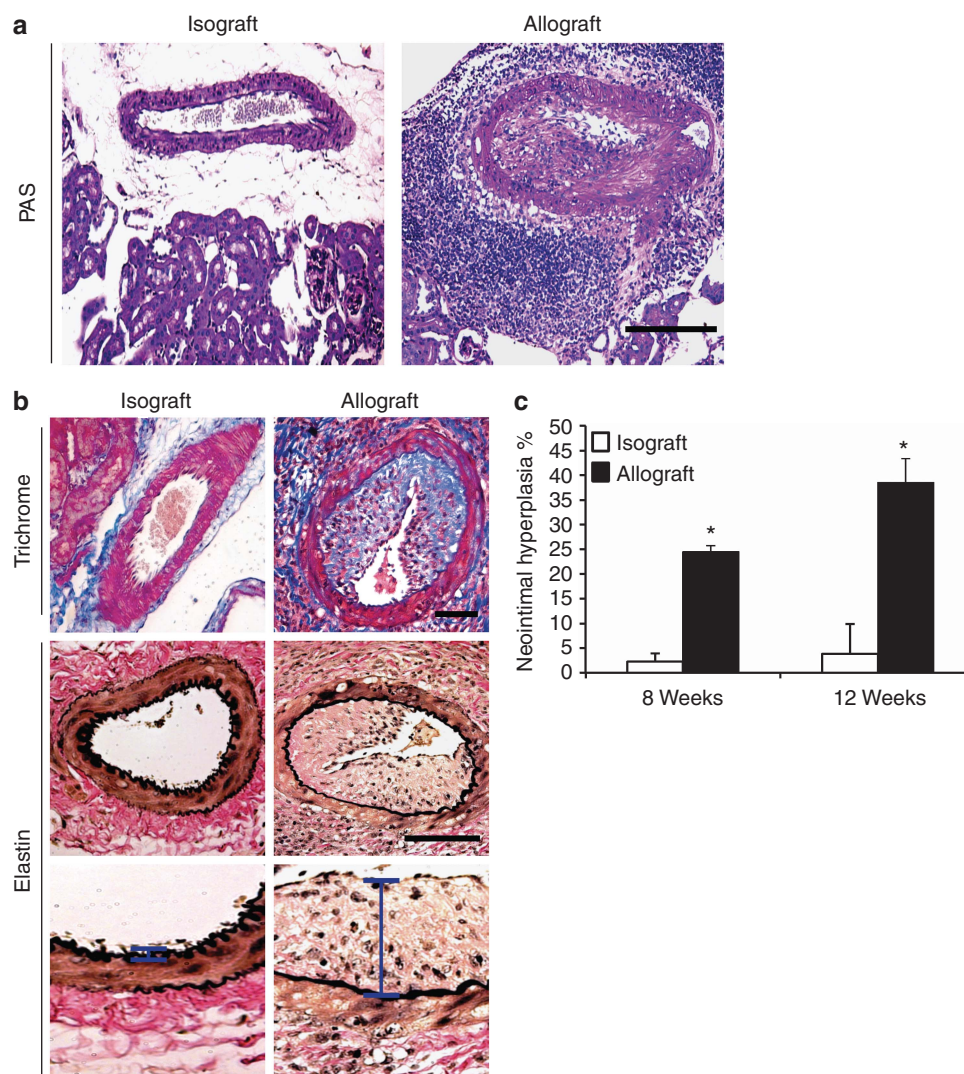


Figure 3 | Perivascular leukocytic infiltrate and neointimal hyperplasia in allografts. (a) Periodic acid-Schiff (PAS) staining of renal allografts depicts significant perivascular mononuclear cell infiltrates and extensive intrarenal arterial neointimal hyperplasia. (b) A representative image of Masson trichrome staining performed on the kidney showing severe neointimal hyperplasia and luminal obliteration that is exclusively found in the allografts (upper panels). Elastin staining was performed (middle panels) to demonstrate the extent of neointimal hyperplasia. Higher-magnification images of the neointimal layer (vertical bar) in the respective groups (lower panels). (c) Quantification of neointimal hyperplasia as described in the methods. * $P < 0.05$. Bar = 100 μm.

Urine was collected via bladder puncture at the time of killing, and the albumin/creatinine ratio was determined as described previously.²¹

HISTOLOGY

For determination of histological changes, kidney grafts were isolated and harvested, fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA), and embedded in paraffin. Five-micrometer serial sections were stained using hematoxylin and eosin, Masson trichrome, periodic acid-Schiff, and elastin staining protocols. Histomorphometric analyses were performed on images acquired with a Digital Module R Leica microscope (Leica, Bannockburn, IL) and IMAGE PRO software (Media Cybernetics, Silver Spring, MD). Neointimal hyperplasia in intrarenal arteries was calculated using the formula [(neointimal hyperplasia area—lumen

area)/neointimal hyperplasia area] × 100 and expressed as a percentage. The area of collagen deposition, stained blue by Masson trichrome staining, was measured by color image analysis software from ten random images (five cortical and five medullary images) from each graft recipient. Five-micrometer frozen sections from five different allograft recipients were used for Immunofluorescence staining of CD11b (Abcam, Cambridge, MA), CD4, and CD8 (Santa Cruz Biotechnology, Santa Cruz, CA).

STATISTICAL ANALYSIS

Data were represented as mean ± s.e.. One-way analysis of variance followed by the Holm-Sidak or Tukey-Kramer test was performed for multiple-group comparisons. Student's *t*-test was used for comparison between two groups. $P < 0.05$ was considered significant.

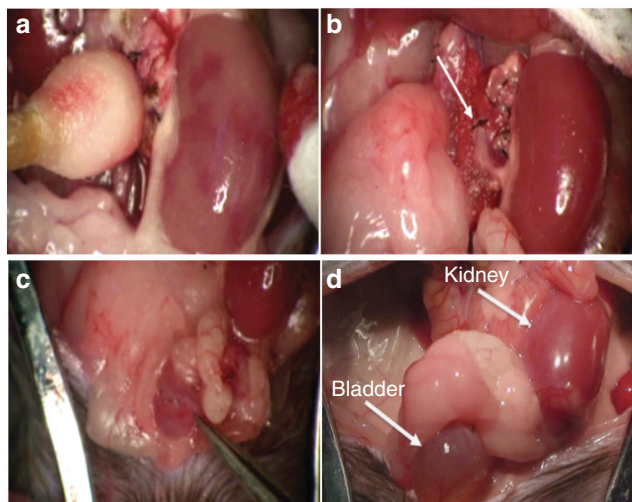


Figure 4 | Mouse orthotopic kidney transplantation.

(a) Transplanted kidney a few seconds following cross-clamp removal. Note the patchy red areas indicating restoration of normal blood flow. (b) The same kidney after complete reperfusion. The upper suture line on the abdominal aorta can also be seen (arrow). (c) The forceps in the lower right corner indicates the suture line where the donor and recipient bladders are joined dome to dome. (d) Gross morphology of a kidney allograft and bladder at 8 weeks after transplantation. Note the presence of urine in the bladder.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Immunofluorescence staining of CD11b (left) and CD8 (right) demonstrates that the majority of perivascular infiltrates are composed of macrophages and CD8⁺ T cells in allografts.

Video file S1. The clip demonstrates the donor kidney along with the ureter and bladder before anastomosis to the recipient.

Video file S2. The clip shows the cross clamp removal following anastomosis. Note the change in color in the graft that manifests full reperfusion.

Supplementary material is linked to the online version of the paper at <http://www.nature.com/ki>

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